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Abstract

This study aimed to evaluate the effects of dietary supplementation with probiotics (PRO), synbiotics (SYN), and SYN + benzoic acid (SYP) on growth performance, stress response, nutrient digestibility, blood parameters, immune status, and intestinal health of weanling pigs. Four hundred weaned pigs (Landrace × Yorkshire × Duroc) were allocated to four treatment groups: control (basal diet), PRO (*Bacillus licheniformis*), SYN (*Bacillus licheniformis* + dietary fiber), and SYP. The feeding trial lasted for 28 days. Notably, pigs in the SYN and SYP groups showed greater average daily gain during phase 1 ($p = 0.038$), phase 2 ($p = 0.008$), and the overall period ($p = 0.011$) compared with those in the control group. Gain-to-feed ratio was also improved during phase 2 ($p = 0.003$) and the overall experimental period ($p = 0.012$). The SYP group demonstrated significantly improved dry matter digestibility during phase 2 ($p < 0.05$). Serum IL-1β concentrations tended to decrease in pigs fed SYN and SYP diets. In addition, SYN and SYP supplementation increased the concentration of the tight junction protein zona occludens-1 (ZO-1) in the jejunum ($p = 0.022$). Histological analysis showed greater villus height in the duodenum ($p = 0.013$) and jejunum ($p = 0.038$) of pigs fed SYN and SYP diets. Overall, dietary supplementation with synbiotics, particularly in combination with benzoic acid, improved growth performance and intestinal health in weanling pigs.

Keywords: weaning pigs, probiotics, benzoic acid, microbiota, immunity

Introduction

Weaning is a critical transition in pig production and is characterized by abrupt dietary and environmental changes that may lead to reduced feed intake, impaired growth performance, and intestinal dysfunction [1,2]. During this period, pigs experience substantial stress, immune challenges, postweaning diarrhea, and intestinal inflammation [3,4]. Traditional-feed antibiotics mitigate these challenges; however, increasing concerns over antimicrobial resistance have driven the search for alternative strategies to enhance gut health and growth performance in weanling pigs [5,6]. Among these alternatives, probiotics, synbiotics, bacteriophages, and organic acids have emerged as promising dietary interventions for improving intestinal function and overall health [7].

Probiotics, including *Bacillus* species, are beneficial microorganisms that modulate the gut microbiota composition, enhance intestinal barrier integrity, and improve nutrient utilization [8-10]. Among these, *Bacillus licheniformis* has been widely used in swine nutrition owing to its ability to produce various digestive enzymes, inhibit pathogenic

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bacteria, and maintain a balanced gut microflora [11,12]. Supplementation with *B. licheniformis* improves nutrient digestibility, enhances intestinal morphology, and upregulates tight junction proteins, thereby promoting gut integrity and reducing intestinal inflammation [8,10–12]. Correspondingly, probiotic supplementation stimulates local immune responses and contributes to the overall health and growth of weanling pigs [13].

Synbiotics are a combination of probiotics and prebiotics that exert synergistic effects by supporting the survival and metabolic activity of beneficial bacteria in the gastrointestinal tract [14,15]. In particular, synbiotic supplementation of *B. licheniformis* with dietary fiber provides dual benefits: *B. licheniformis* improves gut microbial balance and nutrient absorption, whereas dietary fiber serves as a fermentable substrate that generates short-chain fatty acids (SCFA). These SCFAs act as energy sources for enterocytes and play key roles in maintaining intestinal barrier integrity [16-18]. In addition to probiotics and synbiotics, organic acids, such as benzoic acid, have been explored as feed additives for improving gut health in pigs. Benzoic acid reduces intestinal pH, thereby creating an unfavorable environment for pathogenic bacteria, while supporting beneficial microbial populations [19–21]. Moreover, organic acids enhance intestinal barrier function by modulating tight junction proteins and reducing inflammatory responses [5,22,23]. Although the individual effects of probiotics, synbiotics, and organic acids have been extensively investigated, research exploring their synergistic interactions—particularly the specific combination of Bacillus licheniformis-based synbiotics and benzoic acid—remains limited. The novelty of this study lies in its comprehensive evaluation of whether acidifying the gastrointestinal environment with benzoic acid can amplify the prebiotic fermentation and probiotic efficacy of a targeted synbiotic in weaned pigs. Furthermore, by simultaneously assessing multifaceted parameters, including physiological stress markers (e.g., hair cortisol), mucosal immunity, tight junction proteins, and microbiome shifts, this study elucidates the complex interactive mechanisms of these combined additives. Therefore, this study aimed to evaluate the effects of dietary supplementation with probiotics, synbiotics, and synbiotics plus benzoic acid on the growth performance, stress response, nutrient digestibility, blood parameters, immune status, gut integrity, and gut morphology of weanling pigs. By assessing these parameters, the study sought to determine whether these specific additive combinations could serve as effective alternatives to in-feed antibiotics and provide a deeper insight into their synergistic mechanisms of action.

Materials and Methods

Animal and experimental design

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A total of 400 weaned pigs (Landrace × Yorkshire × Duroc) with an average initial body weight (BW) of 6.38 ± 0.13 kg (age: 21 days) were distributed among the following four treatment groups based on BW: a control group fed a basal diet; PRO, basal diet supplemented with probiotics (*Bacillus licheniformis*, 1.0×10^8 colony-forming units [CFU]/g); SYN, basal diet supplemented with synbiotics consisting of *B. licheniformis* (1.0×10^8 CFU/g) and dietary fiber; and SYP, basal diet supplemented with synbiotics consisting of *B. licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid. The synbiotic supplements were experimental products manufactured by WOOGENE B&G (Hwaseong-si, Gyeonggi-do, Republic of Korea) and were not commercially available. In the SYN and SYP treatments, dietary fiber and benzoic acid were incorporated at levels equivalent to 2% of the supplement, and all supplements (PRO, SYN, and SYP) were included in the basal diet at a rate of 0.1% (as-fed basis). Consequently, the final inclusion levels of dietary fiber and benzoic acid in the complete diet were 0.002%. Each treatment was replicated across the four pens, with 25 piglets per pen. The pigs were fed pelleted diets (Table 1) in two phases, phase 1 (days 0–14) and phase 2 (days 15–28), formulated according to the National Research Council [24] guidelines. The pigs were housed in metal pens (3 × 4.8 m) with plastic flooring. Environmental conditions were maintained at an initial temperature of 30°C, which was gradually reduced to 25°C from day 8 onward, with humidity controlled between 61–66%. Each pen was equipped with low-pressure nipple drinkers and self-feeders to ensure *ad libitum* access to water and food.

Experimental sampling

BW was measured at the end of the second and fourth weeks. Growth performance parameters, including average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F), were evaluated at the end of each dietary phase, with adjustments made for mortality. Blood samples were collected from four randomly selected pigs per pen at the end of each phase (days 14 and 28), via jugular vein puncture using anticoagulant-free vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged at 3000 g for 15 min at 4°C and stored at −20°C for subsequent hematological analysis.

Stress indicators

Rectal temperature and respiratory rate were measured twice daily (11:30 and 13:00). Rectal temperature was recorded using a digital thermometer (SK-1260, SATO, Tokyo, Japan), whereas respiratory rate was determined by counting abdominal movements over 1 min with a stopwatch. The average of these readings was used for the statistical

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analysis. Hair cortisol concentrations were assessed using the method described by Nejad et al. [25]. Hair samples were collected from the forehead region on days 0 and 28, stored in aluminum foil, and stored in polypropylene tubes (HM Hyundai Micro) for drying at room temperature. To remove contaminants, samples were washed three times with 5 mL of isopropyl alcohol and dried at 23 ± 1°C for seven days. Cortisol extraction was performed using a methanol dilution, and cortisol levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions.

Digestibility of nutrients

To determine the apparent total tract digestibility (ATTD), chromic oxide (0.25%) was included in the diet as an indigestible marker on days 10–14 and 24–28. Fecal samples were collected on the last two days of each phase, pooled within each pen, and dried in a forced-air oven at 60°C for 72 h. The dried samples were ground using a 1-mm screen in a Wiley mill (Thomas Model 4, Thomas Scientific, Swedesboro, NJ, USA) for further analysis. Analyses were conducted in triplicate following the Association of Official Analytical Collaboration [26] methods: dry matter (DM, method 930.15), crude protein (CP, method 990.03), and ether extract (EE, method 2003.05). The gross energy in the feed and feces was determined using a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL). Chromium concentrations were analyzed via spectrophotometry (Jasco V-650, Jasco Corp., Tokyo, Japan), following the method described by Fenton and Fenton [27].

Sample collection and analysis

Blood samples (10 mL) were collected via jugular vein puncture from four randomly selected pigs per pen on days 14 and 28. Samples were drawn into sodium heparin-containing vacutainer tubes (Becton Dickinson), centrifuged at 3000 g for 15 min at 4°C, and stored at −20°C for further analysis. Biochemical markers, including aspartate transaminase (AST; Kit #08104719190) and alanine transaminase (ALT; Kit #08104697190), creatinine (Kit # 08057532190), and uric acid (Kit # 08058750190) were measured using spectrophotometric kits (TBA-120FR, Toshiba Medical Systems Corporation, Japan) and a Cobas 6000 c501 automatic biochemistry analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Hematological parameters, including white blood cell (WBC) count, red blood cell count, neutrophil count, and lymphocyte count, were assessed using the Hemavet® Hematology System (CDC Technologies, Drew Scientific, Plantation, FL, USA). The cytokines IL-1β (Cat #MBS700738, MyBioSource, San Diego, CA, USA), TNF-α (Cat #MBS2019932, MyBioSource, San Diego, CA, USA), and IL-10 were quantified

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using ELISA kits (Invitrogen, Thermo Fisher Scientific). For intestinal barrier integrity analysis, jejunal tissue samples (~2 cm) were collected from the mid-jejunum immediately after euthanasia, rinsed gently with cold phosphate-buffered saline (PBS), and stored at −80°C until analysis. The frozen tissues were homogenized in ice-cold PBS (1:9, w/v) and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected, and the concentrations of tight junction proteins, ZO-1 (Cat #MBS9310967, MyBioSource, San Diego, CA, USA) and occludin (Cat #MBS740246, MyBioSource, San Diego, CA, USA), were determined using ELISA kits (Invitrogen, Thermo Fisher Scientific). The results were normalized to the total protein concentration, which was measured using the Bradford assay, and expressed as ng/mg total protein.

Intestinal morphology

Intestinal tissue samples were collected from the duodenum, jejunum, and ileum of the pigs. A portion of each sample was frozen in liquid nitrogen and stored at −80°C for future analysis, while the remaining sections (5 cm) were fixed in 10% neutral-buffered formalin for 24 h and transferred to a 70% ethanol solution for preservation. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopic analysis was performed using a Vanox-S Microscope (Olympus Corporation, Lake Success, NY, USA), and image analysis was conducted using SPOT simple imaging software (Diagnostic Instruments, Sterling Heights, MI, USA). Villus height (VH) and crypt depth (CD) were measured in five representative villi and crypts per section.

Microbiota analysis

Fecal samples were collected from representative pigs in each treatment group on days 0 and 28 for microbiota analysis. Metagenomic DNA was extracted from the samples, and the V3–V4 region of the 16S rRNA gene was amplified and sequenced using a next-generation sequencing platform. Sequence data quality control was performed using the DADA2 (v1.18.0) package in R (v4.0.3), with trimming of forward (250 bp) and reverse (200 bp) reads, removal of chimeric sequences, and exclusion of sequences with expected errors ≥2 or a quality score <25. Multiple sequence alignments were performed using MAFFT (v7.475). Alpha diversity (Shannon, Chao1, and Simpson indices) and beta diversity (Bray–Curtis dissimilarity) were calculated using R (v4.4.1). Group differences in diversity and relative abundance at the phylum and genus levels were assessed using t-tests and analysis of variance, whereas differentially abundant taxa were identified using linear discriminant analysis effect size (LEfSe), with a linear

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discriminant analysis (LDA) score threshold of 3.0. Core and unique microbiome analyses were conducted to identify the shared and group-specific taxa.

Statistical analyses

Statistical analysis of the experimental data was performed using the General Linear Model procedure in SAS (SAS Institute. Inc., Cary, NC, USA) using a completely randomized design. The pen was the experimental unit for growth performance and nutrient digestibility, whereas the individual pig was considered an experimental unit for stress indicators, blood parameters, and intestinal morphology. The treatment means were separated using Tukey's multiple range test with $p < 0.05$ indicating statistical significance.

Results

Growth performance

The effects of dietary probiotic and synbiotic supplementation on the growth performance of weanling pigs are presented in Table 2. No substantial differences were observed in the initial or final BW among the treatment groups. During phase 1, ADG was markedly higher in the SYN and SYP groups than in the control group. No considerable differences were observed in ADFI and G:F among the treatments. In phase 2, ADG and G:F were markedly higher in the SYN and SYP groups than in the control group, whereas ADFI remained unaffected. Overall, ADG and G:F were substantially higher in the SYN and SYP groups than in the control group, whereas ADFI showed no notable differences among the treatments.

Stress index

Physiological stress indicators, including respiratory rate, rectal temperature, and hair cortisol concentration, are summarized in Table 3. No notable differences were observed among the treatment groups in phase 1. Similarly, in phase 2, no considerable differences in the respiratory rate, rectal temperature, or hair cortisol levels were observed among the treatments.

Nutrient digestibility

The effects of the dietary treatments on ATTD are shown in Table 4. In phase 1, no notable differences were observed in the ATTD of GE, CP, or EE among the treatments. However, the ATTD of the DM tended to be higher in the SYP

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group than in the control group. In phase 2, the ATTD of the GE, CP, and EE remained unaffected across treatments. However, the ATTD of the DM in the SYP group was markedly higher than that in the control group.

Blood characteristics

The blood parameters are summarized in Table 5. In phase 1, no pronounced differences were observed in terms of WBC and red blood cell count, neutrophil and lymphocyte percentage, and AST, ALT, creatinine, or uric acid levels among the treatments. In phase 2, no notable differences were observed in these blood parameters between the treatment groups.

Immune status and gut integrity

The effects of dietary treatments on immune status and gut integrity are presented in Table 6. In phase 1, no pronounced differences were observed in the blood IL-10 and TNF-α concentrations among the treatment groups. Moreover, IL-1β concentrations tended to decrease in the SYP group compared with that in the control group. In phase 2, IL-10 and TNF-α concentrations showed no substantial changes among treatments, whereas IL-1β concentrations tended to decrease in the SYP group. Intestinal ZO-1 concentration was markedly higher in the SYP group than in the control group (Table 7), whereas no noteworthy differences were observed in the occludin levels across treatments.

Gut morphology

The gut morphology measurements are summarized in Table 8. In the duodenum, VH was substantially higher in the SYN and SYP groups than in the control group, whereas CD remained unchanged. Jejunal VH was markedly greater in the SYN and SYP groups than in the control group, with no pronounced difference in CD. In the ileum, VH and CD showed no considerable differences among the treatments. However, the VH-to-CD ratio (VH/CD) tended to be higher in the PRO, SYN, and SYP groups than in the control group.

Microbiota composition

When the microbiome composition of the treatment groups (PRO, SYN, and SYP) was compared with that of the control at the phylum level (Fig. 1A), feeding trials led to a pronounced reduction in Bacteroidetes (−1.3 to −5.7%) across all experimental groups. In contrast, SYP significantly increased the abundance of Firmicutes (55.7%) and Proteobacteria (3.2%) ($p < 0.01$), whereas the abundance of Firmicutes in the PRO group (52.2%) was lower than that in the control group (55.4%). Spirochaete abundance also decreased significantly in the SYN group (0.16%) compared

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with that in the control group (0.42%) ($p < 0.01$). Subsequently, at the genus level (Fig. 1B), *Ruminococcaceae UCG-002* and *Christensenellaceae R-7* were consistently enriched in all treatment groups, whereas *Prevotellaceae NK3B31*, *Ruminococcaceae UCG-014*, [*Eubacterium*] *coprostanoligenes*, and uncultured *Muribaculaceae* were significantly reduced ($p < 0.05$). In particular, SYN increased the relative abundance of uncultured *Porphyromonadaceae bacterium* (9.9%), *Prevotella* 9 (7.8%), *Ruminococcaceae UCG-002* (3.4%), *Faecalibacterium* (2.6%), *Christensenellaceae R-7* (1.8%), and *Subdoligranulum* (3.2%), while decreasing *Prevotellaceae NK3B31* group (1.5%), *Ruminococcaceae UCG-014* (2.0%), [*Eubacterium*] *coprostanoligenes* group (2.7%), *Muribaculaceae* uncultured bacterium (1.4%), and uncultured *Prevotellaceae* (1.1%). Similarly, SYP significantly increased *Ruminococcaceae UCG-005* (3.4%) and *Christensenellaceae R-7* (1.9%) and decreased *Prevotellaceae NK3B31* (3.5%), *Ruminococcaceae UCG-014* (1.9%), *Coprostanoligenes* (2.6%), and uncultured *Muribaculaceae* (1.4%) ($p < 0.05$). Alpha diversity analysis showed that microbial diversity tended to increase from day 0 to day 28 across all treatment groups, thereby indicating gradual maturation of the gut microbiota after weaning. However, no pronounced differences were observed between treatments at either time point (Fig. 2). In addition, the LEfSe analysis (LDA score > 3.0) identified 34 distinct discriminant taxa in each group (Fig. 3). Subsequently, genera showing significant differences from the control group were selected among the identified 34 taxa. Consequently, *Olsenella* and *Weissella* had a significantly higher relative abundance in the SYP group (Fig. 4A and 4B) and *Catenibacterium* had a significantly higher relative abundance in both the SYN and SYP groups than in the control group (Fig. 4C) ($p < 0.05$). *Oscillospira* had a significantly lower relative abundance in both SYN and SYP groups than in the control group (Fig. 4D) ($p < 0.05$). However, the relative abundances between the SYN and SYP groups showed no considerable differences in any of the selected taxa, including *Olsenella*, *Weissella*, *Catenibacterium*, and *Oscillospira* (Fig. 4).

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Discussion

The improvements in ADG and G:F observed in the SYN and SYP groups suggested that the inclusion of synbiotics had a beneficial effect on the growth performance of weanling pigs. The lack of notable differences in ADFI among treatments indicated that the improved growth efficiency in the SYN and SYP groups was likely attributable to enhanced nutrient utilization rather than increased feed intake.

It should be noted that the inclusion level of benzoic acid used in the present study (0.002%) was substantially lower than the dosages commonly reported to exert direct antimicrobial or acidifying effects in previous studies (typically

0.5–1.0%) [19,20]. At such a micro-dose level, the standalone direct bactericidal or strong acidifying properties of benzoic acid are likely limited. Therefore, the pronounced improvements observed in the SYP group cannot be attributed solely to the classical mechanisms of benzoic acid. Instead, we hypothesize that these improvements reflect synergistic interactions among *Bacillus licheniformis*, dietary fiber, and benzoic acid. Even at low concentrations, benzoic acid may act as a mild modulator that complements the probiotic and prebiotic components, collectively optimizing gut microbial ecology and reinforcing intestinal barrier function [18,22]. These findings align with those of previous studies, indicating that synbiotic supplementation positively influences growth performance in weanling pigs [13,14].

The beneficial effects of this combined approach can be attributed to several integrated mechanisms. Specifically, the probiotic *Bacillus licheniformis* produces extracellular enzymes, such as proteases and amylases, which enhance nutrient digestion and absorption [8,11]. Simultaneously, the fermentation of prebiotic dietary fiber by the gut microbiota generates short-chain fatty acids (SCFAs, such as butyrate, propionate, and acetate), which serve as crucial energy sources for enterocytes, improve intestinal barrier integrity, and reduce inflammation [16]. Furthermore, the micro-dose addition of benzoic acid likely complements this environment by subtly modulating intestinal pH to inhibit pathogenic bacteria (e.g., *Escherichia coli*) while favoring commensals like *Lactobacillus* and *Bifidobacterium*, thereby reducing metabolic energy losses associated with immune activation [5,9,19,28]. Interestingly, although the PRO group did not show notable improvements in growth performance compared with that in the control group, the SYN and SYP groups demonstrated clear advancements. This suggests that the synergistic integration of these three components is necessary to achieve substantial improvements in growth efficiency.

Microbiota analysis revealed that both synbiotic groups (SYN and SYP) exhibited similar shifts, characterized by increased abundances of *Weissella*, *Catenibacterium*, and *Olsenella*, and a reduction in *Oscillospira* compared with that in the control group. *Weissella* produces lactic acid and bacteriocins that contribute to microbial homeostasis and pathogen inhibition [29], whereas *Olsenella* and *Catenibacterium* participate in SCFA synthesis, thereby enhancing intestinal metabolic stability [22]. Conversely, *Oscillospira* has been associated with reduced feed efficiency and slower growth [30]. Thus, synbiotic supplementation contributes to a more stable and metabolically balanced post-weaning gut ecosystem. In particular, the increased abundance of Firmicutes and SCFA-producing families, including

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241 *Ruminococcaceae* and *Christensenellaceae*, in the SYN and SYP groups was consistent with improved fiber
242 fermentation and energy harvesting, contributing directly to the enhanced feed efficiency observed.

243 The observed improvement in DM digestibility in the SYP group further highlights the enhanced nutrient utilization
244 efficiency driven by these combined additives. As established, the extracellular enzymes produced by *B. licheniformis*
245 facilitate the breakdown of complex dietary components [10,12]. This enzymatic action, coupled with the improved
246 microbial balance and favorable hindgut environment, leads to superior absorption and increased feed efficiency
247 without the need for additional feed intake [13,16].

248 Regarding immune status, the tendency for decreased pro-inflammatory IL-1β concentrations in the SYP group
249 suggests anti-inflammatory benefits during post-weaning stress [31,32]. Probiotics like *B. licheniformis* are known to
250 modulate immune function by promoting regulatory T cell activity and enhancing anti-inflammatory cytokines like
251 IL-10 [8,10]. Furthermore, the enrichment of SCFA-producing taxa in these groups actively downregulates
252 inflammatory responses via increased butyrate availability [16,18]. This enhanced microbial ecosystem also positively
253 influenced gut barrier function, as evidenced by the increased blood ZO-1 concentrations in the SYP group [6,33].
254 The structural integrity of tight junction proteins is likely supported by the combined stabilization of gut permeability
255 mediated by both SCFA production and organic acid modulation [21,23]. The absence of differences in occludin
256 concentration suggests that the structural core of tight junction proteins remains stable.

257 The improved intestinal absorptive capacity, indicated by the increased VH in the duodenum and jejunum of the
258 SYN and SYP groups, provides morphological evidence of enhanced gut health [7]. The accelerated enterocyte
259 proliferation and villus development are direct functional outputs of the synergistic mechanisms mentioned earlier,
260 particularly the increased SCFA production in the hindgut which fuels enterocyte differentiation [2,11,34]. The
261 absence of pronounced changes in CD across treatments suggests that cell turnover rates remained stable. The trend
262 toward an increased VH/CD ratio in the ileum in the PRO, SYN, and SYP groups further supports the notion that
263 dietary supplementation contributes to improved gut morphology.

264 In the SYP group, the pronounced shift toward a community enriched in fiber-fermenting, SCFA-producing bacteria
265 (e.g., UCG-002, UCG-005, and *Christensenellaceae* R-7) directly supports the observed morphological and
266 immunological improvements, as these taxa degrade complex polysaccharides and strengthen tight junction integrity

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[1,4,17,18]. The concurrent decline in taxa associated with proinflammatory responses and inefficient energy utilization (e.g., *Prevotellaceae* NK3B31, *Ruminococcaceae* UCG-014, and [*Eubacterium*] *coprostanoligenes*) further validates the metabolically efficient nature of this microbial shift [3,35,36]. In addition, the detection of unique taxa, such as *Methanobrevibacter*, suggests improved fermentation efficiency via hydrogen removal, while the enrichment of lactic acid bacteria (*Weissella*, *Olsenella*, and *Parabacteroides*) enhances colonization resistance against pathogens [15,22]. Collectively, these compositional shifts confirm that the specific combination of *Bacillus licheniformis*, prebiotic fiber, and benzoic acid successfully creates a stable, metabolically efficient, and anti-inflammatory gut ecosystem [8,11,12,14,20,21,23].

Conclusion

This study demonstrates that dietary supplementation with synbiotics and benzoic acid improves the growth, nutrient digestibility, intestinal morphology, and immune status of weanling pigs. Although probiotics alone did not confer considerable benefits, the synergistic effects of *Bacillus licheniformis* with dietary fiber (SYN) enhanced growth efficiency and villus development, whereas the addition of benzoic acid (SYP) further improved DM digestibility and modulated the gut microbiota toward a more beneficial SCFA-producing profile. These findings suggest that combining synbiotics with organic acids provides a multifaceted strategy for enhancing gut health and productivity in piglets, thereby offering a viable alternative to in-feed antibiotics. Accordingly, future studies should investigate the long-term effects of these additives on lifetime productivity, microbiota stability, and resilience to pathogenic challenges.

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389 **Tables**

Table 1. Experimental basal diet

Item	Phase 1	Phase 2
Ingredients (%)		
Corn	33.39	48.22
Soybean meal	15.20	18.10
Whey	15.00	10.00
Lactose	12.00	6.00
Fishmeal	5.00	3.00
SDPP	5.00	2.00
Animal fat	4.59	3.99
Sugar	6.00	4.00
L-Lys (78%)	0.35	0.49
DL-Met (99%)	0.13	0.18
L-Thr (99%)	0.11	0.17
L-Trp (100%)	0.12	0.31
Limestone	1.10	1.10
Monocalcium phosphate	0.88	1.31
Salt	0.50	0.50
ZnO	0.30	0.30
Vitamin premix[1]	0.11	0.11
Mineral premix[2]	0.22	0.22
Total	100.00	100.00
Chemical composition (%)		
Metabolizable energy	3400.00	3350.00
Crude protein	20.00	18.00
Crude fat	6.38	5.94

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Ash	5.76	5.59
Calcium	0.85	0.80
Phosphorus	0.62	0.59
Lys	1.53	1.40
Met + Cys	0.87	0.79
Phe	0.90	0.82

[1]Supplied per kg of diet: 16000 IU vitamin A (palmitate), 2.00 mg vitamin B_1 (thiamin), 5.00 mg vitamin B_2 (riboflavin), 2.00 mg vitamin B_6 (pyridoxine), 0.03 mg vitamin B_{12} (cyanocobalamin), 25.00 mg niacin, 0.40 mg folic acid, 0.05 mg biotin, 5.00 mg ethoxyquin, 2000 IU vitamin D_3 (cholecalciferol), 75.00 mg vitamin E (dl-α-tocopheryl acetate), 2.00 mg vitamin K_3 (menadione).

[2]Supplied per kg of diet: 100 mg Fe, 6 mg Cu, 4 mg Mn, 0.3 mg Se, 0.14 mg I, 0.25 mg Co; SDPP, spray dried plasma protein.

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Table 2. Effects of dietary probiotic and synbiotic supplementation on growth performance in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Initial BW[6] (kg)	6.360	6.400	6.390	6.370	0.030	0.642
Final BW (kg)	15.700	15.930	16.150	16.200	0.120	0.131
Phase 1 (days 0–14)						
ADG[7] (g)	284.000[b]	292.000[ab]	302.000[a]	304.000[a]	5.810	0.038
ADFI[8] (g)	414.000	418.000	428.000	427.000	9.560	0.426
G:F[9]	0.687	0.698	0.707	0.713	0.010	0.121
Phase 2 (days 15–28)						
ADG (g)	384.000[b]	389.000[ab]	395.000[a]	397.000[a]	5.870	0.008
ADFI (g)	592.000	598.000	595.000	596.000	10.220	0.496
G:F	0.648[b]	0.651[ab]	0.663[a]	0.665[a]	0.010	0.003
Overall (day 0–28)						
ADG (g)	334.000[b]	340.000[ab]	348.000[a]	350.000[a]	4.690	0.011
ADFI (g)	503.000	508.000	511.000	511.000	6.170	0.385
G:F	0.664[b]	0.670[ab]	0.682[a]	0.685[a]	0.010	0.012

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test.

[6] BW, body weight

[7] ADG, average daily gain

[8] ADFI, average daily feed intake

[9] G:F, feed efficiency.

391 [a,b] Means within a row with different superscripts differ significantly ($p < 0.05$).

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Table 3. Effects of dietary probiotic and synbiotic supplementation on stress indices and hair cortisol in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Phase 1 (day 14)						
Respiratory rate (breaths/min)	21.480	19.560	21.010	20.070	1.080	0.232
Rectal temperature (℃)	37.370	37.430	37.350	37.460	0.180	0.892
Hair cortisol (pg/mg)	136.650	131.470	134.570	132.440	4.960	0.235
Phase 2 (day 28)						
Respiratory rate (breaths/min)	20.820	20.300	19.860	21.080	1.270	0.757
Rectal temperature (℃)	37.630	37.640	37.650	37.480	0.220	0.997
Hair cortisol (pg/mg)	135.330	139.230	142.940	138.240	4.650	0.334

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

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Table 4. Effects of dietary probiotic and synbiotic supplementation on nutrient digestibility in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Phase 1 (day 14)						
DM[6] (%)	78.550	78.950	79.280	79.400	0.250	0.051[†]
GE[7] (%)	80.700	81.060	81.590	81.460	0.370	0.119
CP[8] (%)	78.640	78.790	78.940	78.930	0.250	0.465
EE[9] (%)	70.950	70.590	70.700	71.330	0.380	0.642
Phase 2 (day 28)						
DM (%)	78.430[b]	78.670[ab]	79.200[a]	79.240[a]	0.200	0.013
GE (%)	80.750	81.050	81.420	81.130	0.330	0.146
CP (%)	78.380	78.960	79.020	78.960	0.350	0.125
EE (%)	71.260	70.880	71.570	70.640	0.300	0.170

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

[6] DM, dry matter

[7] GE, gross energy

[8] CP, crude protein

[9] EE, ether extract.

[†] Indicates a tendency ($0.05 \leq p < 0.10$).

[a,b] Means within a row with different superscripts differ significantly ($p < 0.05$).

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Table 5. Effects of dietary probiotic and synbiotic supplementation on blood characteristics in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Phase 1 (day 14)						
WBC[6] (10^6/μL)	16.960	18.170	18.630	18.650	1.380	0.452
RBC[7] (10^6/μL)	6.530	5.780	5.780	5.960	0.750	0.671
Neutrophil (10^3/μL)	8.020	9.730	8.150	8.450	1.530	0.522
Lymphocyte (10^3/μL)	10.130	9.250	10.540	9.990	0.680	0.315
AST[8] (U/L)	60.740	71.930	63.120	70.580	7.010	0.424
ALT[9] (U/L)	1006.500	927.500	983.000	996.800	81.530	0.559
Creatinine (μmol/L)	88.870	86.780	88.110	88.240	2.830	0.667
Uric acid (μmol/L)	4.620	4.870	4.810	4.760	0.820	0.569
Phase 2 (day 28)						
WBC (10^6/μL)	17.790	18.540	17.560	18.050	1.240	0.665
RBC (10^6/μL)	6.520	6.450	5.800	6.450	0.670	0.581
Neutrophil (10^3/μL)	7.780	8.580	7.660	8.220	1.740	0.764
Lymphocyte (10^3/μL)	9.250	10.170	10.570	10.150	0.630	0.233
AST (U/L)	64.080	73.140	67.620	70.560	9.300	0.244
ALT (U/L)	1085.800	924.700	965.600	926.900	118.190	0.409
Creatinine (μmol/L)	86.860	90.250	82.51	91.250	3.590	0.127
Uric acid (μmol/L)	4.150	3.950	5.090	4.550	0.580	0.146

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

[6] WBC, white blood cell

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[7] RBC, red blood cell

[8] AST, aspartate aminotransferase

[9] ALT, alanine aminotransferase.

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Table 6. Effects of dietary probiotic and synbiotic supplementation on immune status in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Phase 1 (d 14)						
IL-1β[6] (pg/mL)	59.630	56.630	53.480	52.980	2.300	0.059[†]
IL-10[7] (pg/mL)	131.220	130.650	135.620	135.120	4.400	0.365
TNF-α[8] (pg/mL)	157.880	160.440	149.490	148.390	5.630	0.155
Phase 2 (day 28)						
IL-1β (pg/mL)	56.080	55.040	51.200	50.860	1.910	0.051[†]
IL-10 (pg/mL)	132.020	129.540	130.850	130.100	5.360	0.642
TNF-α (pg/mL)	160.310	160.790	150.710	148.790	5.010	0.166

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

[6] IL-1β, interleukin-1β

[7] IL-10, interleukin-10

[8] TNF-α, tumor necrosis factor-α.

[†] Indicates a tendency ($0.05 \leq p < 0.10$).

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Table 7. Effects of dietary probiotic and synbiotic supplementation on jejunal tight junction protein concentrations in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Jejunum						
ZO-1[6] (ng/mg protein)	10.320[b]	13.890[ab]	15.410[a]	15.900[a]	1.830	0.022
Occludin (ng/mg protein)	3.510	3.900	3.630	3.720	0.440	0.743

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

[6] ZO-1, zona occludens-1

[a,b] Means within a row with different superscripts differ significantly ($p < 0.05$).

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Table 8. Effects of dietary probiotic and synbiotic supplementation on gut morphology in weanling pigs

Item	Control	PRO[1]	SYN[2]	SYP[3]	SEM[4]	*p*-value[5]
Duodenum						
VH[6] (μm)	541.980[b]	558.150[ab]	560.740[a]	566.810[a]	5.320	0.013
CD[7] (μm)	313.430	314.880	311.220	311.040	8.300	0.907
VH/CD	1.730	1.770	1.800	1.820	0.040	0.271
Jejunum						
VH (μm)	500.980[b]	513.160[ab]	519.590[a]	518.310[a]	6.090	0.038
CD (μm)	289.440	297.170	289.180	300.700	7.020	0.464
VH/CD	1.730	1.730	1.800	1.730	0.040	0.243
Ileum						
VH (μm)	464.130	468.930	471.320	472.490	5.500	0.445
CD (μm)	321.200	311.460	314.020	312.860	5.080	0.194
VH/CD	1.450	1.510	1.500	1.510	0.030	0.081

[1] PRO, *Bacillus licheniformis* (1.0×10^8 colony-forming units [CFU]/g);

[2] SYN, *Bacillus licheniformis* (1.0×10^8 CFU/g) and dietary fiber;

[3] SYP, *Bacillus licheniformis* (1.0×10^8 CFU/g), dietary fiber, and benzoic acid;

[4] SEM, standard error of means;

[5] Statistical analysis was performed as described for Table 1.

[6] VH, villus height;

[7] CD, crypt depth.

[a,b] Means within a row with different superscripts differ significantly ($p < 0.05$).

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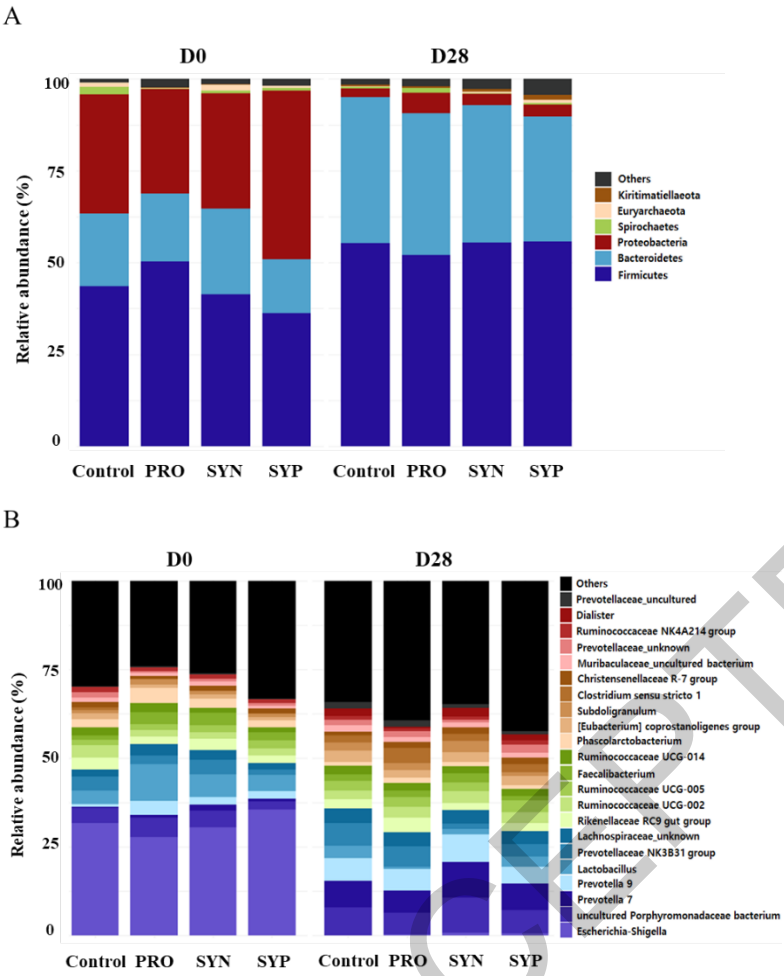

Fig. 1. Relative abundance of fecal microbiota at the (A) phylum and (B) genus level at day 0 and day 28. Values represent mean relative abundance across treatment groups. PRO, *Bacillus licheniformis*; SYN, *Bacillus licheniformis* and dietary fiber; SYP, *Bacillus licheniformis*, dietary fiber, and benzoic acid.

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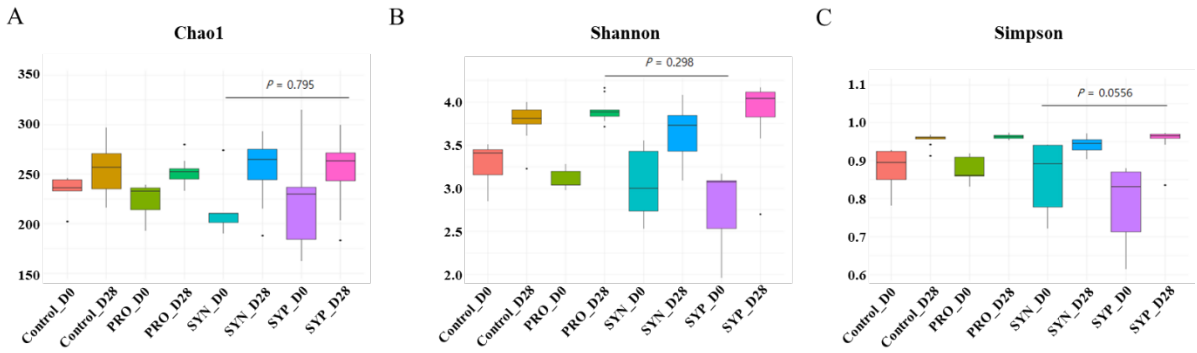

Fig. 2. Alpha diversity indices of fecal microbiota at Day 0 and Day 28. (A) Chao1, (B) Shannon, and (C) Simpson (C). PRO, *Bacillus licheniformis*; SYN, *Bacillus licheniformis* and dietary fiber; SYP, *Bacillus licheniformis*, dietary fiber, and benzoic acid.

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Group	Feature	LDA (log10)	pvalue	padj
Control	*Prevotellaceae NK3B31 group*	4.5	0.010	0.010
	Lactobacillus	4.2	0.008	0.008
	Butyrivibrio	3.2	0.002	0.002
	[Eubacterium] xylanophilum group	3.0	0.046	0.046
PRO	*Clostridium sensu stricto 1*	4.2	0.049	0.049
	Succinivibrionaceae UCG-001	4.0	0.014	0.014
	Succinivibrio	3.9	0.001	0.001
	Treponema 2	3.8	0.003	0.003
	Alloprevotella	3.7	0.013	0.013
	Prevotella 1	3.7	0.003	0.003
	Prevotella 2	3.6	0.000	0.000
	Prevotellaceae UCG-003	3.6	0.001	0.001
	Lachnospiraceae NK4A136 group	3.5	0.017	0.017
	Oscillospira	3.4	0.033	0.033
	Erysipelotrichaceae UCG-004	3.4	0.020	0.020
	Clostridium sensu stricto 6	3.1	0.012	0.012
	Bacteroidales_uncultured_bacterium	3.1	0.024	0.024
	Lachnospira	3.1	0.001	0.001
	WCHB1-41_uncultured rumen bacterium	3.0	0.010	0.010
SYN	*Dialister*	4.1	0.026	0.026
	Subdoligranulum	4.1	0.035	0.035
	Escherichia-Shigella	3.9	0.028	0.028
	Catenibacterium	3.5	0.020	0.020
	Collinsella	3.5	0.001	0.001
	Lachnospiraceae NK3A20 group	3.3	0.001	0.001
SYP	*Prevotellaceae_unknown*	3.9	0.025	0.025
	Olsenella	3.9	0.012	0.012
	Methanobrevibacter	3.6	0.012	0.012
	Weissella	3.6	0.009	0.009
	Staphylococcus	3.3	0.004	0.004
	Parabacteroides	3.3	0.024	0.024
	Campylobacter	3.3	0.012	0.012
	WCHB1-41_uncultured_bacterium	3.1	0.016	0.016
	Prevotellaceae UCG-001	3.1	0.008	0.008

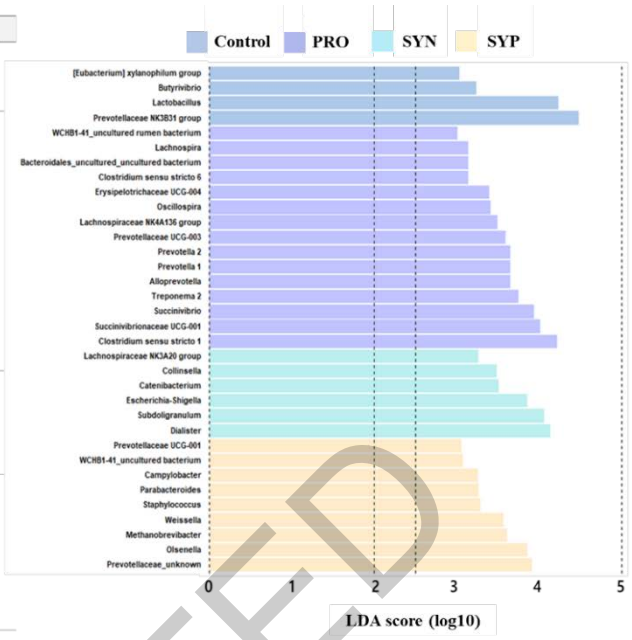

Fig. 3. Linear discriminant analysis effect size (LEfSe) identifying differentially abundant taxa among treatments at day 28. Only taxa with linear discriminant analysis (LDA) scores >3.0 are shown. PRO, *Bacillus licheniformis*; SYN, *Bacillus licheniformis* and dietary fiber; SYP, *Bacillus licheniformis*, dietary fiber, and benzoic acid.

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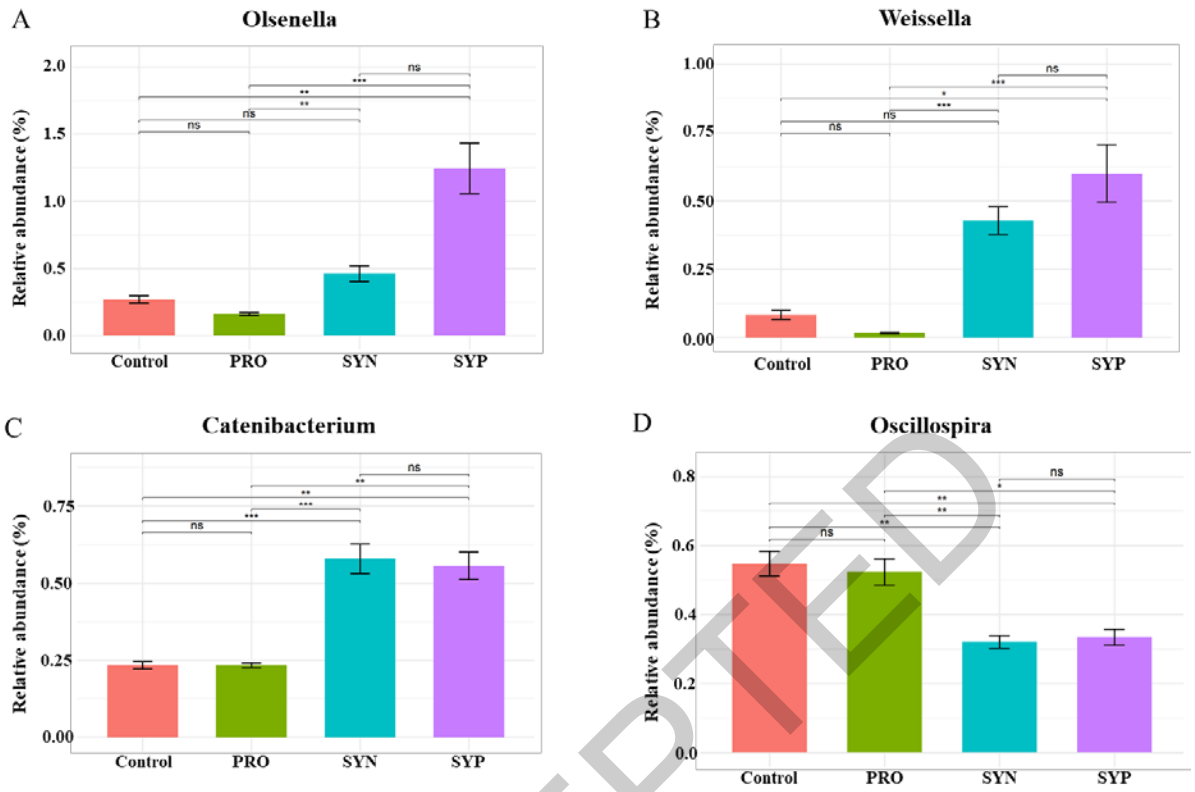

Fig. 4. Relative abundance of specific fecal microbes among treatment groups at day 28. (A) *Olsenella*, (B) *Weissella*, (C) *Catenibacterium*, (D) *Oscillospira* (D). Values represent mean ± SEM. PRO, *Bacillus licheniformis*; SYN, *Bacillus licheniformis* and dietary fiber; SYP, *Bacillus licheniformis*, dietary fiber, and benzoic acid; SEM, standard error of meas. Statistical significance: ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$(Data were analyzed using the Kruskal–Wallis test, followed by Dunn's post hoc test).

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